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STRUCTURE OF THE NERVOUS SYSTEM OF THE AURICULARIA LARVA OF *PARASTICOPUS CALIFORNICUS*

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Abstract

The structure and organization of the larval nervous system of the holothurian, *Parasticopus californicus,* is described using glyoxylic acid-induced fluorescence, indirect immunofluorescence with antibodies against serotonin, and transmission and scanning electron microscopy. Tracts of catecholaminergic axons are located at the base of the ciliary bands and catecholaminergic nerve cell bodies are dispersed along the length of the ciliary bands. Clusters of catecholaminergic cells form a ganglion on the lower lip of the larva and a ganglion of serotonergic cells is located at the anterior tip of the larva. Serotonergic cells are scattered throughout the apical portion of the larva in the epidermis. Axon tracts identified only with TEM are located in the esophagus associated with the circumesophageal muscles. The neuroanatomy of the auricularia shares several features with larval forms of the other classes of echinoderms.

Introduction

Holothurians typically have either planktotrophic development in which a feeding auricularia larva metamorphoses into a non-feeding doliolaria larva, or lecithotrophic development in which the embryo forms directly into a doliolaria (reviewed by Hyman, 1955). Forms which develop directly into the pentactula or juvenile without an intervening larval stage are also known. There are numerous accounts of the development of holothurians which describe the general features of larval structure (MacBride, 1914; Mortensen, 1931, 1937, 1938; Hyman, 1955; Inaba, 1957; Oguru, 1974). As well, Strathmann (1971) has given a comprehensive account of feeding in the auricularia and there are descriptions of metamorphosis and settlement of doliolariae (Ohshima, 1921; Inaba, 1957; Young and Chia, 1982). However, detailed knowledge of larval structure and the processes of metamorphosis for this class of echinoderms are fragmentary (reviewed by Hyman 1955, Strathmann, 1978).

The information available on other forms of echinoderm larvae, especially echinoids, is far greater. This is due in part to the difficulties encountered in obtaining gametes and raising holothurian embryos and larvae. Until recently techniques for obtaining fertilizable ova from most species of sea cucumber did not exist. Maruyama (1980, 1985), Kishimoto *et al.* (1982), and Smiley (pers. comm.) have developed techniques for the use of radial nerve extracts and disulphide reducing agents which permit detailed studies of holothurian larvae grown in culture (Maruyama, 1980).

Here we describe the organization and structure of the nervous system of an auricularia. Aspects of the neuroanatomy of four of the distinctive larval forms of echinoderms are now available, permitting a comparison of the general features of neural organization and the cytological characteristics of neural tissues of larval echinoderms.

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Materials and Methods

Larvae were reared using the techniques outlined by Strathmann (1968). Adults were collected subtidally in the vicinity of Friday Harbor, Washington, and Victoria, British Columbia. Gonadal tubules were dissected from females and put in seawater containing an extract of radial nerve prepared from *P. californicus* (the generous gift of Mr. Scott Smiley). After 1–2 hours germinal vesicles had begun to break down and eggs were expelled from the gonadal tubules. Eggs were transferred to culture dishes and a dilute suspension of sperm, prepared from dissected testes, was added. Water was changed every 24–48 hours and cultures fed a suspension of *Dunaliella salina.* Sixteen- to twenty-seven-day-old auriculariae were used for all experiments and observations. Larvae metamorphosed to doliolariae beginning about 25 days after fertilization and settled as pentactulae at about 30 days.

Specimens were prepared for glyoxylic acid-induced fluorescence following the procedures detailed in Burke and Gibson (1986). For immunofluorescence larvae were fixed in 4% paraformaldehyde in filtered seawater (FSW) for 2.5 h at room temperature and stored in FSW containing 0.01 *M* sodium azide. Fixed larvae were incubated in phosphate buffered saline (PBS) containing 10% goat serum and 0.3% Triton X-100 for 30 min to reduce non-specific binding and increase permeability. Specimens were incubated in rabbit anti-serotonin (diluted 1/90 in PBS) (Immuno Nuclear Corp.) for 16 h at 4°C, rinsed in PBS, and incubated in FITC conjugated goat anti-rabbit IgG (1/16 PBS) for 1 h at room temperature before being viewed and photographed with a Zeiss Universal microscope fitted for epifluorescence. The specificity of the primary antibody was assessed by pre-absorbing a 1/45 dilution of it with an equal volume of 1 mg/ml serotonin for 30 min.

Larvae were prepared for transmission electron microscopy (TEM) following the methods outlined in Burke (1985). Specimens for scanning electron microscopy were fixed in 2.5% glutaraldehyde buffered in 0.1 *M* sodium cacodylate, pH 7.3, to which several drops of 4% OsO_4 had been added. After 5 min in this solution larvae were transferred to buffered glutaraldehyde containing no OsO_4. After several months storage in this fixative larvae were rinsed well with 0.1 *M* sodium cacodylate and fixed with 1% OsO_4 in 1.25% $NaHCO_3$ for 1 hour before dehydration in a graded series of ethanol and critical point drying. Specimens were sputter coated with gold and viewed on a JEOL JSM-35 scanning electron microscope.

Results

The auricularia larva of *P. californicus* is typical of holothurian auriculariae described previously (Mortensen, 1937, 1938; Strathmann, 1971). It has a single, sinuate circumoral ciliary band that is its principal feeding and locomotory organ (Fig. 1). The mouth, which is overhung by the pre-oral hood, is surrounded by a second ciliary band, the adoral ciliary band. Larvae used in this study, 16 to 27 days, increased slightly in size, developed more pronounced looping of the circumoral ciliary band, and elaborated the axohydrocoel and spherules, but otherwise changed little in form.

Ciliary bands in the auricularia are thickened regions of the epidermis with a row of densely packed, simple cilia. The bands range from 10 to 20 μm in thickness and typically have a number of evenly spaced mesenchyme cells associated with the blastocoelar surface (Fig. 2). The mesenchyme cells extend numerous filiform processes into the blastocoel.

In glyoxylic acid treated preparations, fluorescent tracts are associated with all of the ciliary bands of the auricularia. The fluorescent tracts are 2 to 3 μm thick and situated medially at the base of the ciliary band (Figs. 3, 4, 5). At intervals ranging

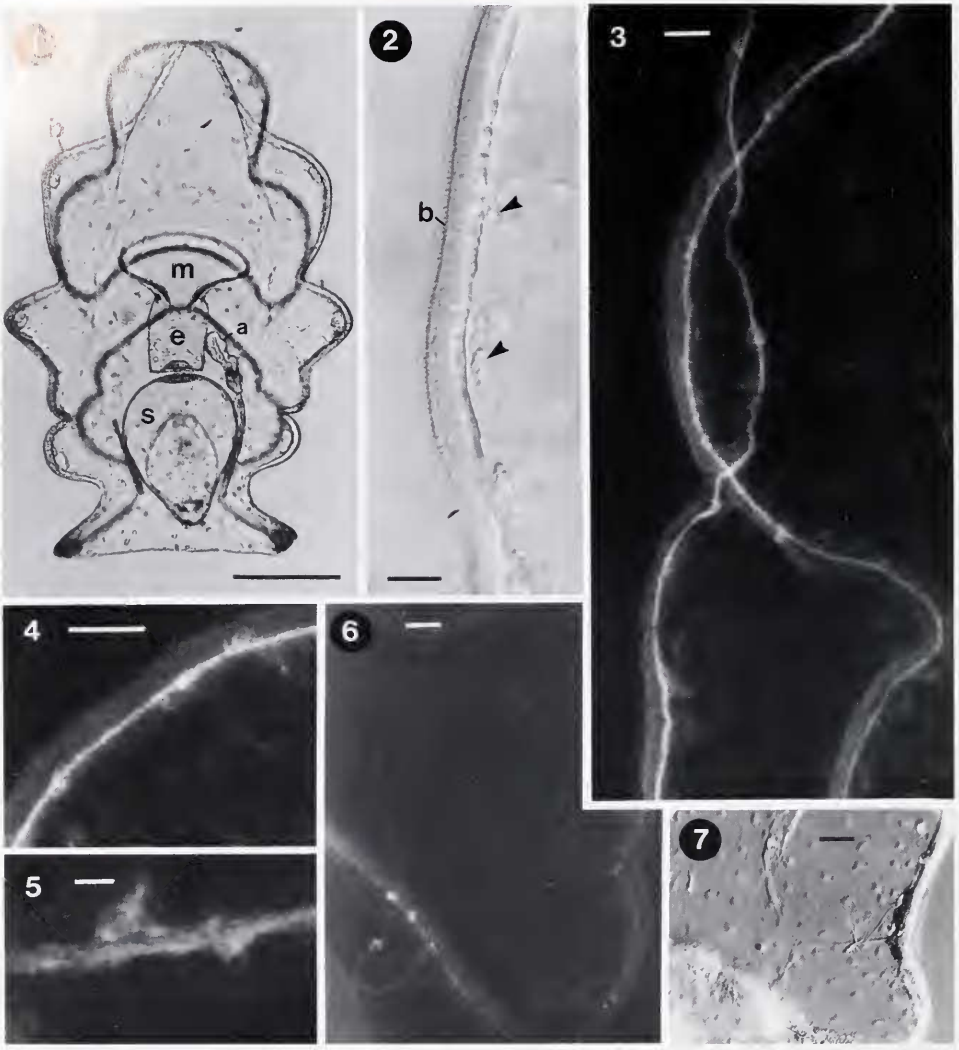

FIGURE 1. Auricularia larva of *Parasticopus californicus* 18 days after fertilization. a, axohydrocoel; b, circumoral ciliary band; e, esophagus; m, mouth; s, stomach. Bar = 100 μm.

FIGURE 2. Detail of circumoral ciliary band (b). Arrows indicate mesenchymal cells associated with the ciliary band. Nomarski differential contrast (DIC) optics. Bar = 20 μm.

FIGURE 3. Glyoxylic acid-induced fluorescence of catecholamines. A region of the circumoral ciliary band showing the fluorescent tracts situated at the base of the ciliary band. Bar = 20 μm.

FIGURE 4. Fluorescent tract and cell bodies associated with the circumoral ciliary band. Glyoxylic acid-induced fluorescence. Bar = 20 μm.

FIGURE 5. Detail of fluorescent tract showing individual strands and cell bodies. Glyoxylic acid-induced fluorescence. Bar = 5 μm.

FIGURE 6. Fluorescent tracts and associated cell bodies that lie lateral to the larval mouth. Glyoxylic acid-induced fluorescence. Bar = 20 μm.

FIGURE 7. Triradiate tracts and cell bodies that lie lateral to the mouth in a live auricularia of *P. californicus*. Nomarski DIC optics. Bar = 20 μm.

from 50 to 150 μm along the length of the fluorescent tracts are 5 to 10 μm diameter thickenings that appear to be cell bodies (Figs. 4, 5). At higher magnification the fluorescent tracts can be resolved as numerous strands (Fig. 5). On both sides of the larva, lateral to the mouth, there are fluorescent tracts that are not associated with a ciliary band (Fig. 6). These tracts appear to join the lateral tracts associated with the pre-oral and post-oral ciliary bands. Often one or more cell bodies are associated with these branches. With Nomarski differential interference contrast optics, these bilaterally paired triradiate tracts and the cells associated with them can be resolved in live specimens (Fig. 7).

The mouth of the larva is an elliptical opening about 125 μm wide and 75 μm high in the middle of the oral field (Fig. 1). The adoral ciliary band is 6–7 μm thick around the perimeter of the mouth except in the notch that forms the lower lip where the band thickens to about 10 μm (Fig. 8). Fluorescent tracts occur throughout the adoral ciliary band of glyoxylic acid treated specimens in a similar location as described for those of the circumoral ciliary band. On the lower lip there is typically an aggregation of from 9 to 15 brightly fluorescent cell bodies (Figs. 9, 10). The cells are irregular in shape and up to 12 μm in diameter. Concentrations of cells are greatest on the sides of the notch of the lower lip and appear scattered up the adoral ciliary band.

At the apical tip of the larva the circumoral ciliary bands of the left and right sides approach each other but remain separated by 10 to 15 μm of epidermis. In glyoxylic acid preparations a fluorescent tract underlying the epidermis joins the fluorescent tracts of the ciliary bands (Fig. 11). Cell bodies were never observed in this region using this technique. However, in indirect immunofluorescent preparations using anti-serotonin antibodies, a ganglion of 10 to 12 cells was shown to lie beneath the epidermis at the apical tip of the larva (Figs. 12, 13). The cells are 10 to 12 μm in diameter, polygonal in outline, and multipolar (Fig. 13). The cells of the apical ganglion are interconnected by numerous axonal and dendritic processes up to 2 μm in diameter. As well, a number of immunoreactive cells that are similar in appearance are scattered along the apical circumoral ciliary band. No other immunoreactive cells were identified throughout the larval body. In control experiments in which the primary antibody was pre-absorbed with serotonin, fluorescence in these cells was reduced to nearly background levels.

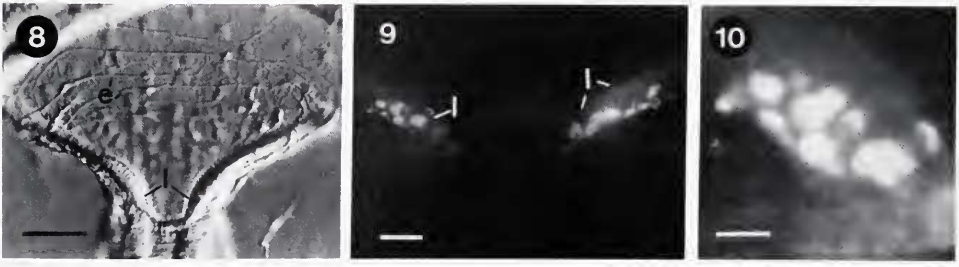

FIGURE 8. Thickened regions of the adoral ciliary band which form the lower lip (l) of the larval mouth of an auricularia of *Parasticopus californicus*. e, esophageal muscles. Nomarski DIC optics. Bar = 20 μm.

FIGURE 9. Fluorescent cell bodies within the lower lip (l). Gloxylylic acid-induced fluorescence. Bar = 20 μm.

FIGURE 10. Detail of irregularly shaped cell bodies in the lower lip. Glyoxylic acid-induced fluorescence. Bar = 10 μm.

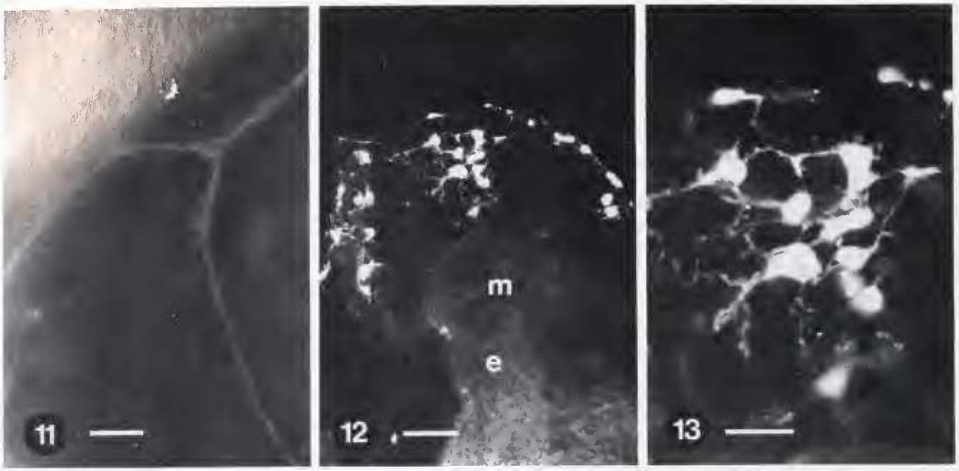

FIGURE 11. Fluorescent tract that joins the right and left circumoral ciliary band tracts at the apical tip of the auricularia larva of *Parasticopus californicus*. Glyoxylic acid-induced fluorescence. Bar = 20 μm.

FIGURE 12. Indirect immunofluorescence with anti-serotonin showing the ganglion at the apical end of an auricularia larva. e, esophagus; m, mouth. Bar = 40 μm.

FIGURE 13. Detail of the multipolar cells and cell processes of the apical ganglion. Indirect immunofluorescence using anti-serotonin. Bar = 10 μm.

Ultrastructure

The thickened epithelium of the ciliary bands of the auricularia are comprised of numerous flask-shaped, ciliated cells with their tapered apices aggregated to form the densely ciliated band (Fig. 14, inset). The ciliated cells have a single cilium at their apical end, a centrally located nucleus, and contain various organelles including mitochondria, Golgi bodies, lysosomes, and axially oriented microtubules. The ciliated cells extend the full thickness of the band and their bases aggregate around a single tract of axons (Fig. 14). Occasionally in sections of the ciliary band, nerve cells extending axonal processes into the axonal tract can be identified. These cells typically have an elaborate, electron-dense Golgi body surrounded by numerous 0.1 to 0.4 μm diameter vesicles (Fig. 15). The nerve cells are about 12 μm in length and appear to extend to the outer surface of the larva. The axons range in size from 0.2 to 1.0 μm in diameter and indeterminate length (Figs. 16, 17). The number of axons within the tract varies from 8 to about 40. The clear cytoplasm of the axons contains numerous microtubules, mitochondria, and vesicles. Typically the vesicles range in size from 0.05 to 0.10 μm and contain either fine granular material or an electron-dense core. Vesicles of both types can be found within the same axon (Figs. 16, 17).

The adoral ciliary band on the lower lip can be seen in scanning electron micrographs to form two ciliated palp-like prominences (Fig. 18). In section, the palps are a simple columnar epithelium made up of ciliated cells, interspersed with nerve cells (Fig. 19). At the base of the epithelium are numerous axons aggregated into tracts that extend the length of the esophagus (Fig. 20). Nerve cells of the adoral ciliary band are elliptical in outline and are distinguished by a prominent, centrally located Golgi body which is surrounded by numerous 0.05 to 0.1 μm vesicles. The apical half of these cells contain 0.2 to 0.6 μm vacuoles which are irregular in outline and contain electron-dense material condensed into a loose irregular meshwork (Fig. 21). Throughout its

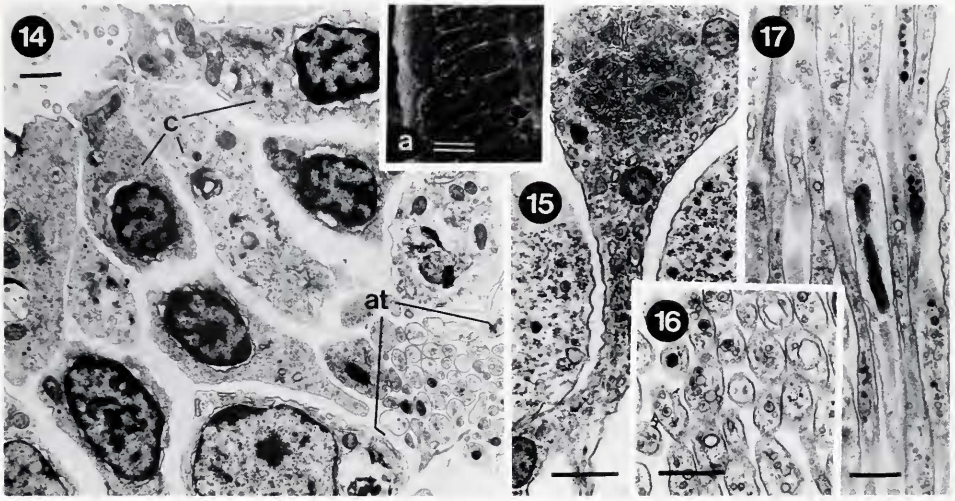

FIGURE 14. Transmission electron micrograph (TEM) of a transverse section through the circumoral ciliary band of a 23-day-old auricularia showing the ciliated cells (c) and the tract of axons (at) that lies at the base of the ciliated cells. Bar = 1 μm. a. (inset) scanning electron micrograph (SEM) showing the densely ciliated circumoral ciliary band. Bar = 0.5 μm.

FIGURE 15. Section through a basal portion of a nerve cell giving rise to axonal processes in the ciliary band. TEM. Bar = 0.5 μm.

FIGURE 16. Transverse section through axonal tract of the circumoral ciliary band showing vessicles containing fine granular material and electron-dense material. TEM. Bar = 0.5 μm.

FIGURE 17. Longitudinal section through the axonal tract of the circumoral ciliary band. TEM. Bar = 1 μm.

length, axon tracts also course around the perimeter of the esophagus. The axons occur in groups of up to 20–25, typically underlie the epithelial cells, and do not cross the basal lamina (Figs. 22, 23).

Nerve cells outside the ciliary bands occur at the apical end of the larva in regions adjacent to the ciliary bands (Figs. 24, 25). The nerve cells are distinguished by the prominent, electron-dense Golgi body and numerous 0.05 to 0.15 μm vesicles. Axons, in loosely organized tracts underlying the epidermis, are scattered throughout this region (Fig. 26).

The cells within the blastocoel that are associated with the ciliary bands (Fig. 2) are characterized ultrastructurally by large vacuoles that contain a variety of materials (Fig. 27). In some sections these appeared to be phagosomes containing amorphous material, or whole, necrotic cells. The remainder of the cytoplasm of these cells typically contains small cisternae of rough endoplasmic reticulum, mitochondria, and lysosomes.

DISCUSSION

The principal behaviors of the auricularia are swimming and feeding (Strathmann, 1971). The ciliary bands are arranged so that a larger proportion of ciliary beat is directed posteriorly and the larva moves with its anterior end foremost. As particles pass over the ciliary band, there are localized reversals of ciliary beat which deflect the particles into the oral field. Food particles are transported to the mouth by currents produced either by portions of the lateral band or by the adoral ciliary band. There are three means by which larvae are able to reject particles: (*i*) not retaining particles

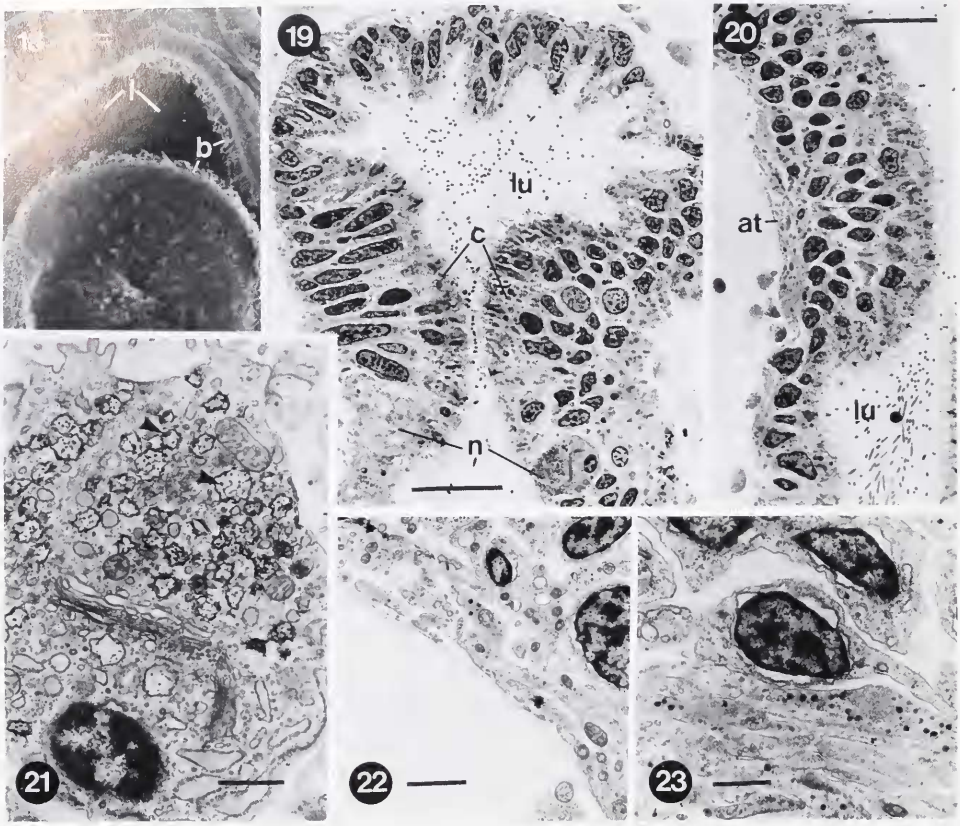

FIGURE 18. SEM of the adoral ciliary band (b) showing the palp-like thickenings (l) of the lower lip of a 23-day-old auricularia of *Parasticopus californicus*. Bar = 20 μm.

FIGURE 19. Transverse section through the lower lip showing the ciliated cells (c) and nerve cells (n) of the adoral ciliary band. lu, lumen of the esophagus. TEM. Bar = 10 μm.

FIGURE 20. Longitudinal section through the thickened region of the adoral ciliary band of the lower lip of an auricularia of *P. californicus*. at, axonal tract; lu, lumen of the esophagus. TEM. Bar = 10 μm.

FIGURE 21. Section through a nerve cell from the adoral ciliary band. Arrows indicate irregularly shaped vacuoles containing electron-dense material. TEM. Bar = 1 μm.

FIGURE 22. Section through axonal tracts at the base of the esophageal epithelium. TEM. Bar = 2 μm.

FIGURE 23. Detail of axonal tracts within the thickened epithelium of the lower lip. TEM. Bar = 1 μm.

as they pass over the circumoral ciliary band; (*ii*) overall reversal of the direction of ciliary beat, causing particles to be cleared from the oral field and the larva to be propelled backwards; and (*iii*) reverse peristalsis of the esophageal muscles causing regurgitation of the contents of the esophagus. Strathmann (1971) noted that regurgitation may be accompanied by ciliary reversal of the circumoral ciliary band as well as reversal of the adoral ciliary band. When larvae encounter an obstacle they reverse the direction of ciliary beat and back away.

Although this behavioral repertoir is not complex, it does show coordination of effectors (ciliary reversals accompanying esophageal regurgitation) and responses of effectors some distance from the point of stimulation (obstacle avoidance). These observations have led several authors to speculate that auriculariae possess a nervous

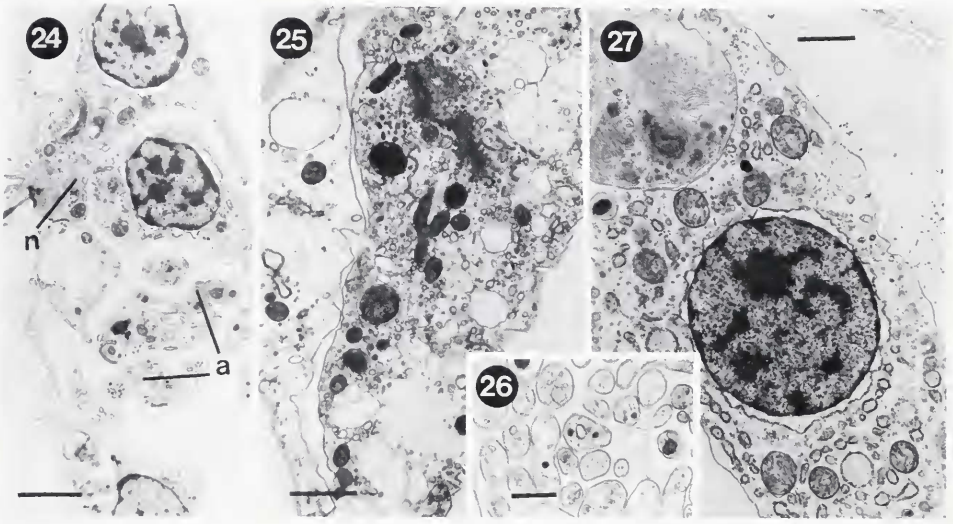

FIGURE 24. Transverse section through the epidermis at the apical end of an auricularia larva showing nerve cells (n) and axons (a) that are not within the circumoral ciliary band. TEM. Bar = 2 μm.

FIGURE 25. Detail of nerve cell from the epidermis at the apical end of a larva. TEM. Bar = 1 μm.

FIGURE 26. Detail of axons underlying the epidermis at the apical end of a larva. TEM. Bar = 0.5 μm.

FIGURE 27. Section through a blastocoelar mesenchyme cell associated with the circumoral ciliary band. TEM. Bar = 1 μm.

system (Mortensen, 1920; Garstang, 1939; Strathmann, 1971, 1975). Strathmann (1971) observed that in the presence of excess $MgCl_2$, an inhibitor of synaptic transmission in some animals, overall reversals of the ciliary band, rejection of particles from the esophagus, and localized reversal associated with feeding were blocked.

The principal effector organs of the larva are the ciliated cells of the ciliary bands and the musculature of the esophagus. The neural components observed in this study are associated with larval effectors and probably function primarily in coordinating swimming and feeding behaviors of the larva. Conceivably the nervous system mediates overall ciliary reversals and coordinates reverse peristalsis of the esophageal musculature with reversals. It is also possible that the nervous system mediates the localized ciliary reversals involved with particle capture. The sparse distribution of nerve cells within the ciliary band would argue against such a hypothesis as it would be expected that the spacing of the cells would approximate the length of band involved in a localized reversal. Although this distance is not known for sure (Strathmann, 1971), it would appear to be much shorter than the 50 to 150 μm observed between nerve cells of glyoxylic acid treated specimens.

The nervous system of the echinopluteus has been described by Ryberg (1977) and Burke (1978, 1981, 1983a, b) and is thought to consist of tracts of axons associated with the ciliary bands and esophagus along the length of which are interspersed nerve cell bodies. An apical neuropil and ganglion on the lower lip of the pluteus has also been described (Burke, 1983a). The nervous system of the bipinnaria and brachiolaria larva of asteroids includes axon tracts associated with the ciliary bands and the esophagus (Barker, 1978; Burke, 1983c). Two types of nerve cells are interspersed along the length of the ciliary band, and ganglia and neuropil are associated with the lower lip of the mouth and the brachiolarian arm. The larval nervous system of the doliolaria

larva of the crinoid, *Florometra serratissima,* is described by Chia *et al.* (1986) as consisting of tracts of axons associated with the ciliary bands and an extensive apical ganglion associated with the larval adhesive organ. The neural organization of the ophiopluteus of *Ophiopholis aculeata* is similar to that of the other larval echinoderms with axonal tracts associated with the ciliary bands and an oral ganglion (Burke, unpub. obs.).

There are several similarities in the organization of these nervous systems. All the forms have axon tracts associated with the ciliary bands. In the feeding larval forms (asteroids, echinoids, and holothuroids) nerve cells are also associated with the ciliary band. In the non-feeding doliolaria the axons are associated with the ciliary bands, but no nerve cells were identified within the ciliary bands and the axons also appear to form a general plexus underlying the epidermis. All the echinoderm larvae so far examined appear to have an apical nerve center which typically takes the form of a neuropil. However in holothurians and crinoids nerve cell bodies are associated with the apical nerve center and as such it has been described as a ganglion. Only in the feeding larval form has a ganglion on the lower lip been described.

These similarities in the nervous systems of echinoderm larvae are probably homologous characteristics which underline the affinities between the larval forms. Although the larval forms of echinoderms are different in general appearance, the similarities in the manner in which they feed (Strathmann, 1971, 1975) and the organization of their ciliary bands and nervous system argues for a close relationship amongst them (Dan, 1957). In constructing a phylogeny for echinoderms, if adult characteristics and the fossil record form the basis of the phylogeny (Fell and Pawson, 1966) or if rRNA sequences are used (R. A. Raff, pers. comm.), the similarities between the echinopluteus and ophiopluteus and between the auricularia and bipinnaria must be attributed to convergence. The apparent similarity in their tissue level of organization leaves the principal difference between the forms the presence or absence of a pluteus-like larval skeleton. This suggests that the convergence is based on a relatively small number of characteristics that may have been acquired or lost independently of other larval characteristics.

Specimens prepared for immunofluorescence with anti-serotonin revealed a distinct population of nerve cells not visualized with the glyoxylic acid-induced fluorescence. Ultrastructurally these cells have several characteristics consistent with them being nerve cells, but none which distinguished them from the nerve cells revealed with glyoxylic acid. Glyoxylic acid-induced fluorescence has been suggested to be specific for dopaminergic nerves (Grace and Bunny, 1980; Sharpe and Atkinson, 1980), however, Keenan and Koopowitz (1981) have presented evidence that glyoxylic acid will induce fluorescence of L-dopa, L-dopamine, L-tyrosine, and norepinephrine with emittence spectra that are indistinguishable. In preparations of echinoplutei the pattern of nerves revealed with anti-dopamine antibodies is identical to that observed with glyoxylic acid induced fluorescence (Bisgrove, 1985). Although this does not assure specificity of glyoxylic acid-induced fluorescence, it does corroborate the interpretation that the cells are dopaminergic.

It is possible that the nerves so far identified with immunofluorescence and histochemistry do not comprise the entire nervous system of the larva. Neither technique was able to provide an image of the nerve tracts identified with TEM in the esophagus. In the echinopluteus, these nerves are immunoreactive with antibodies against gamma-aminobutyric acid (Bisgrove, 1985). The mesenchyme cells within the blastocoel associated with the ciliary band appear to be homologous with the mesenchyme cells of the echinopluteus suggested to be nervous by Ryberg (1977). There are however, no cytological characteristics that suggest they may function as nerve cells. As techniques

for the identification of nervous tissues become more refined, the full extent of the larval nervous system will become clearer.

The auricularia of *P. californicus* metamorphoses to a doliolaria after about three weeks. The transformation is radical, involving an overall shrinking in the size and rearrangement of the circumoral ciliary band into four separate bands of cilia surrounding the barrel-shaped larva (reviewed by Hyman, 1955). The doliolaria is transformed gradually and directly in about a week into the pentactula which settles. It is not known how much of the larval nervous system is carried over into the adult form, though as the ciliary bands are ultimately resorbed, it is likely that at least a portion of the nervous system is destroyed as well. In other planktotrophic echinoderm larvae it appears that the nervous system of the larva is destroyed during metamorphosis (Chia and Burke, 1978). It is possible that the neural tissues are specialized for control and coordination of larval tissues and play little or no role in the development of the adult nervous system. The complexity of the nervous system of the auricularia appears greater than is necessary for control of the relatively simple larval effectors, suggesting that other roles for the nervous system may exist. Neurosecretory and neuroendocrine processes may exist which function in controlling the developmental events of metamorphosis as has been hypothesized for other larval forms (Hadfield, 1978; Chia, 1978; Burke, 1983a, d). However, other than the relatively rapid and coordinated onset of metamorphosis, at present there are no data to support such a hypothesis.

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